COMPARATIVE ANATOMY AND MORPHOLOGY OF Vitis vinifera (Vitaceae) SOMATIC EMBRYOS FROM SOLID- AND LIQUID-CULTURE-DERIVED PROEMBRYOGENIC MASSES

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Ontogeny of somatic embryos of grapevine (Vitis vinifera) produced from solid- and liquid-culture-derived proembryogenic masses (PEM) was compared using light and scanning electron microscopy. Somatic embryos produced from solid-medium-derived PEM (SPEM) had large cotyledons, little or no visible suspensor structure, and a relatively undeveloped concave shoot apical meristem, whereas those from liquid-medium-derived PEM (LPEM) had smaller cotyledons, a distinct suspensor, and a flat-to-convex shoot apical meristem. The convex shoot apical meristem in LPEM-derived somatic embryos formed as early as the heart stage of development; it was 4–6 cell layers deep and rich in protein. Suspensors persisted in fully developed and mature LPEM-derived somatic embryos. The SPEM-derived somatic embryos exhibited dormancy, as do mature zygotic embryos, which also have a rudimentary suspensor, whereas LPEM-derived embryos were not dormant. We hypothesize that the presence of a persistent suspensor in LPEM-derived somatic embryos modulates development, ultimately resulting in rapid germination and a high plant-regeneration rate.

Key words: cell culture; embryogenesis; grapevine; somatic embryogenesis; Vitaceae; Vitis vinifera.

Although somatic and zygotic embryos are nearly identical in structural and functional characteristics (Goldberg et al., 1989, 1994; Gray and Purohit, 1991a), the ontogeny of somatic embryos tends to be more variable (Litz and Gray, 1992). Somatic embryos of most species, including grapevine (Vitis spp.), tend to exhibit several typical morphological abnormalities such as variation in shape, size, and cotyledon number (Goebel-Tourand et al., 1993; Gray, 1995; Jayasankar et al., 2002). In most species, somatic embryos are larger than zygotic embryos, and their regeneration rates are lower. For instance, in Vitis rupestris Scheele, only 3% of somatic embryos were capable of developing into complete plants, although 27% had shoot and root apices (Faure, 1990).

Grapevine somatic embryogenesis, first reported by Mullins and Srinivasan (1976), has become commonplace for several genotypes (Gray, 1995). In most reports, initiation and maintenance of embryogenic cultures were accomplished by growth on solidified medium, and plant-regeneration rates were often low (<20%). Recently, we demonstrated an embryogenic liquid culture system in which somatic embryos differ morphologically from their solid-medium-derived counterparts and exhibit a higher plant-regeneration rate (>60%) (Jayasankar et al., 1999). In the present study, we show the ontogenic pattern to be set very early (i.e., in proembryogenic masses [PEM]), because PEM initiated in solid or liquid culture systems then plated onto solid medium produced somatic embryos with characteristic morphological and developmental differences. Anatomical and morphological studies have proven useful in understanding somatic and zygotic embryo development (Altamura et al., 1992; Gray, 1995; Faure et al., 1996a, b). Hence, we employ the same approach to compare somatic embryogenesis from liquid- and solid-medium-derived PEM (LPEM and SPEM, respectively) to investigate reasons for differential ontogeny and plant-regeneration efficiency.

MATERIALS AND METHODS

Culture maintenance—Embryogenic cultures of Vitis vinifera L. ‘Chardonnay’ (Clone 15) and ‘Thompson Seedless’ were induced from anthers and leaves, respectively, and maintained either on solid medium as described by Gray (1995) or in liquid culture and maintained as PEM as described by Jayasankar et al. (1999). To produce somatic embryos for study, PEM from solid and liquid culture systems were plated onto solidified X6 medium as previously described (Li et al., 2001). Zygotic embryos also were excised from fully mature seeds of ‘Chardonnay’ for comparative study. Embryos at various developmental stages were selected using a stereomicroscope and fixed immediately for light and scanning electron microscopy.

Scanning electron microscopy (SEM)—All fixation and rinse solutions were buffered to pH 6.8 with 0.05 mol/L Sorenson’s phosphate buffer and kept at 4°C. Unless otherwise specified, all fixation and dehydration steps were done on ice. Embryos were fixed in cold 3% glutaraldehyde and incubated overnight at 4°C. After rinsing with buffer (three times), embryos were fixed in 1% osmium tetroxide for 30 min and then rinsed three times. Embryos were then dehydrated in a graded ethanol series (15% increments every 30 min). After two changes (15 min each) of 100% ethanol, embryos were subjected to critical-point drying, mounted on stubs, sputtered with gold-palladium, and immediately observed with a Hitachi model S330 SEM.
Light microscopy—Embryos were fixed in glutaraldehyde and serially dehydrated in ethanol as described. A trace amount of safranin was added to the first 100% ethanol rinse, and specimens were incubated at room temperature for 15 min to introduce color into the normally translucent embryos for visualization in the resin for micro-orientating and sectioning. After two quick rinses with 100% ethanol to remove excess safranin, embryos were transferred to cold 100% ethanol for another 15 min. Dehydrated embryos were embedded in flat molds in JB4-Plus plastic resin (Polysciences, Pennsylvania, USA), as per manufacturer’s instructions. The embryos were carefully oriented in the molds to facilitate subsequent median longitudinal sectioning. Small blocks of resin, approximately 1–4 mm³, each containing an embryo or cluster of embryos, were excised from the molds and attached to the end of solidified resin blanks with Superglue in an orientation for cutting of median longitudinal sections. Specimens were reoriented several times during facing and sectioning to obtain median longitudinal sections. Sections approximately 5 μm thick were cut with glass knives and secured to slides by heating at 70°C. The sections were stained with periodic acid–Schiff’s reagent (PAS) to contrast polysaccharides (e.g., cell walls and amyloplasts) and counter stained with naphthol blue black to visualize proteins.

RESULTS

Embryogenic cultures—Regardless of solid or liquid medium origin, an embryogenic culture typical of grapevine developed when PEM were transferred to and grown on solidified X6 medium. Because embryogenic cultures grew nonsynchronously, somatic embryos in different stages of development were present at any given time (Figs. 1, 3, 10). Somatic embryos were white and opaque (Fig. 1). Despite the general similarity among embryogenic cultures, SPEM- and LPEM-derived somatic embryos differed in their structural and functional characteristics.

SPEM-derived somatic embryos—The SPEM-derived somatic embryos typically developed with enlarged (often supernumerary) cotyledons (Figs. 1, 5). When isolated from the underlying cell mass, a narrow pointed attachment reminiscent of a suspensor was apparent (Fig. 2). As a result of this limited attachment, such embryos were sessile with respect to underlying tissue (Fig. 3). Repetitive embryogenesis occurred in the immediate subtending tissue (Fig. 4, arrows), resulting in typical groupings of somatic embryos, which often appeared to be fused to each other at their bases, but were, in fact, attached only through this embryogenic tissue. The shoot apical meristem of solid-medium-derived somatic embryos typically was reduced in size, concave in cross section, and composed of four or more cell layers (Figs. 12, 13). The root apical meristem and embryonic vasculature did not differ substantially from SPEM-derived somatic embryos (Figs. 12, 14).

LPEM-derived somatic embryos—The LPEM-derived somatic embryos differed from their SPEM-derived counterparts in that they had an enlarged suspensor apparatus, which was apparent from the earliest stages of development (Fig. 8) and persistent through maturity (Figs. 9–12). Such embryos developed without attachment/fusion to each other and often became perched well above subtending tissue (Figs. 8, 9, 10). The suspensor was multiseriate, often exceeding seven cell layers in width (Fig. 11). In early stages, a clear demarcation between the suspensor region and embryo proper could not be established. Like SPEM-derived somatic embryos, LPEM-derived embryos also often had supernumerary cotyledons, but they were distinctly smaller in size (Fig. 12). The shoot apical meristem was better developed than that of SPEM-derived embryos, being flat-to-convex in cross section and composed of four or more cell layers (Figs. 12, 13). The root apical meristem and embryonic vasculature did not differ substantially from SPEM-derived somatic embryos (Figs. 12, 14).

Development of somatic embryos—Figures 15–24 reconstruct the developmental sequence of LPEM-derived somatic embryos, based upon individually selected specimens. Early stages of embryogenesis occurred rapidly from LPEM. Small globular somatic embryos were observed within 2 wk after plating LPEM onto solid medium. As embryos reached the globular stage, they protruded above subtending tissue on an elongate suspensor (Fig. 15), and the distinction between the suspensor and the embryo proper became clear. As the embryo body continued to enlarge, it initially maintained a radial symmetry (Figs. 15–18). Embryos up to this stage were approximately 500 μm or less in length, of which more than 60% was the suspensor. Assumption of bilateral symmetry occurred as early as 3 wk after plating LPEM onto solid medium, as evidenced by differential enlargement from two distinct sites of accelerated growth; this signaled the start of cotyledon development and resulted in the appearance of a concave dimple in the distal end of the embryo (Figs. 19, 20, arrows). Further cell divisions and enlargement led to elongation of the embryo body and early definition of the cotyledons (Fig. 20, arrow). The cotyledonary poles rapidly outgrew the central region, leaving a distinct notch between them (Fig. 21, arrow). This corresponded to a typical “heart stage” of development. Also, at this stage, the dimple became convex in many LPEM-derived somatic embryos from cell divisions in the shoot apical meristem (Fig. 22, arrow). The cotyledons differentiated further, completely enveloping the shoot apical meristem, and the hypocotyl continued to enlarge as embryos reached the “torpedo stage” of development. Embryos at this stage were 1.0–2.5 mm in length. Within 5 wk of plating, well-defined, morphologically correct dicotyledonous somatic embryos were apparent (Fig. 23).

Mature LPEM-derived somatic embryos (Fig. 24) typically differed from SPEM-derived somatic embryos (Fig. 25) by their large, persistent suspensors and smaller cotyledons. The LPEM-derived somatic embryos were nearly identical in morphology to zygotic embryos in that they both had heart-shaped cotyledons typical of grapevine; however, somatic embryos were noticeably larger than zygotic embryos, which tended to be flattened (compare Figs. 25, 26). As with some SPEM-derived somatic embryos, zygotic embryos had a rudimentary suspensor (Fig. 26). The relative length of hypocotyl-to-cotyledon varied among the three embryo types. In SPEM-derived somatic embryos, the cotyledons were longer than the hypocotyls. In LPEM-derived somatic embryos, the hypocotyls were longer than the cotyledons, whereas in zygotic embryos the lengths were approximately equal.

DISCUSSION

In SPEM-derived culture material, typical development of grapevine somatic embryos was nearly identical to that of zygotic embryos (Gray, 1995). Somatic embryos have more morphological variation, such as pluricotyly, than zygotic embryos, which are smaller and flattened, probably from differences in the in vitro and in vivo (seed) environments. In the seed, the developing zygotic embryo is physically compressed (Gray...
Figs. 15–26. Developmental morphology of liquid-medium-derived somatic embryos (15–24) compared with solid-medium-derived somatic embryo (25) and zygotic embryo (26). 15. Initially, the suspensor (Su) is the most prominent part of the developing embryo apparatus. 16. As the embryo body (Em) begins to differentiate, it becomes more obvious. 17–18. Continued development results in a late-stage, globular embryo. 19. A concave dimple (arrow) in the distal end of the embryo is the first sign of cotyledons and development of shoot apical meristem. 20. The embryo body begins to elongate and the dimpled area becomes more prominent (arrow). 21. Cotyledon enlargement (arrow) results in a distinct cleft. 22. The dimpled area (arrow) becomes convex as the shoot apical meristem differentiates. 23. Continued growth results in an elongated embryo with cotyledons that enclose the shoot apical meristem. 24. A mature liquid-medium-derived somatic embryo exhibits smaller cotyledons and a persistent suspensor (Su), when compared to solid-medium-derived somatic embryo (25) or a zygotic embryo (26). The cotyledons of solid-medium-derived somatic embryo and zygotic embryo are typically heart-shaped; however, the zygotic embryo is smaller and laterally flattened in comparison. Bars = 500 μm.

and Purohit, 1991a). Developmentally, both SPEM-derived somatic embryos and their zygotic counterparts exhibit physiological dormancy; they do not germinate to undergo plant development without a dormancy-breaking pretreatment.

It was surprising to discover that two morphologically distinct types of grapevine somatic embryos could be produced on solid medium; the only apparent difference was the initial culture environment in which the PEM were grown. When compared to somatic embryos from SPEM, somatic embryos from LPEM possessed a large, persistent suspensor, smaller cotyledons, and a more anatomically defined shoot apical meristem. Developmentally, LPEM-derived somatic embryos also differed in that they did not exhibit dormancy (i.e., they germinated readily without a pretreatment to break dormancy) and produced a higher percentage of plants compared to SPEM-derived somatic embryos (>60% vs. <20%). It is tempting to link structural differences to the observed developmental and physiological differences between solid- and liquid-medium-derived somatic embryos.

In contrast to the persistent and robust suspensor of LPEM-derived somatic embryos, the typical suspensor in zygotic embryos is programmed to die when the embryo proper reaches the torpedo stage (Yeung and Meinke, 1993). The suspensors in LPEM-derived somatic embryos are often long, multiser-
iate, and relatively massive. Their cytoplasm-rich cells suggest that they are metabolically active, perhaps in supplying nutrients or modulating the hormonal balance in the developing somatic embryos (Souter and Lindsey, 2000). In contrast, the suspensors in mature zygotic embryos, when detected, are only a few cells long. The cells are often highly vacuolated, suggesting that they are not cytologically active. The suspensor originally was thought to be an embryonic accessory that was necessary for positioning the developing embryo in the embryo sac. However, studies with common bean (Phaseolus vulgaris L.) have shown that it is necessary for active protein synthesis in developing zygotic embryos (Brady and Walthall, 1985). Later, Nagl et al. (1991) demonstrated the synthesis of storage proteins in the suspensor cells of beans.

We earlier observed that LPEM-derived somatic embryos germinate precociously without undergoing the dormancy typical of grapevine zygotic embryos (Jayasankar et al., 1999), whereas somatic embryos derived from SPEM exhibit dormancy (Gray, 1989; Gray and Purohit, 1991a, b), and require a pretreatment to germinate (Gray and Mortensen, 1987; Gray and Purohit, 1991b). Such dormancy in grapevine somatic embryos is attributed to ABA accumulation, which typically reaches a peak during maturation (Rajasekaran et al., 1982). However, exogenous GA3-induced grapevine somatic embryo germination, and the concentration of GA-like compounds also increased during cold stratification (Takeno et al., 1983; Pearce et al., 1987). The persistent suspensor in LPEM-derived somatic embryos may be a reason for the lack of dormancy and concomitant precocious germination. Suspensors also are a site of gibberellin synthesis. Very high levels of GA-like substances have been found in suspensors of Trapaeanum (Picciarelli et al., 1984), Cytisus (Picciarelli et al., 1991), and Phaseolus (Piaggio et al., 1989). Studies in P. coccineus L. have shown that GA moves from suspensor to the embryo proper during embryo maturation (Alpi et al., 1975, 1979). We hypothesize that gibberellins are produced abundantly and continuously in the suspensors of LPEM-derived somatic embryos and then are transported to the embryo proper, leading to precocious germination.

Because certain nutrients and growth regulators are specifically produced only in the suspensor, lack of an adequate suspensor may deprive somatic embryos of growth factors needed for proper shoot apical meristem development. This lack is suggested by the poor apical meristem development exhibited by SPEM-derived somatic embryos, which have premature vacuolation in the meristematic region. Embryos with such a defective meristem are not likely to develop into a complete plant. Premature vacuolation also has been implicated in the failure to develop a functional meristem in carrot (Nickle and Yeung, 1993) and canola (Yeung et al., 1996). Such poor development of the meristematic region in many SPEM-derived somatic embryos may be a factor leading to low efficiency of plant regeneration.

Plant regeneration using somatic embryogenesis is necessary to produce novel genotypes after genetic manipulation. This necessity is particularly evident considering that many plant transformation systems, including grapevine (e.g., Li et al., 2001), employ embryogenic culture systems as a source of target cells. However, little attention has been paid to the developmental processes leading to regeneration of a whole plant from a single somatic cell. Yeung and Stasolla (2000) proposed that the capacity of a somatic embryo to regenerate into a plant largely depends on the quality of its shoot apical meristem. Our research indicates that this viewpoint suggests that proper development of the shoot apical meristem depends, in turn, on the presence of certain embryonic organs, particularly the suspensor.

LITERATURE CITED


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